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The effects of co-colonising ectomycorrhizal fungi on mycorrhizal colonisation and sporocarp formation in *Laccaria japonica* colonising seedlings of *Pinus densiflora*

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Abstract

Forest trees are colonised by different species of ectomycorrhizal (ECM) fungi that interact competitively or mutualistically with one another. Most ECM fungi can produce sporocarps. To date, the effects of co-colonising fungal species on sporocarp formation in ECM fungi remain unknown. In this study, we examined host plant growth, mycorrhizal colonisation, and sporocarp formation when roots of *Pinus densiflora* are colonised by *Laccaria japonica* and three other ECM fungal species (*Cenococcum geophilum*, *Pisolithus* sp., and *Suillus luteus*). Sporocarp numbers were recorded throughout the experimental period. The biomass, photosynthetic rate, and mycorrhizal colonisation rate of the seedlings were also measured at 45 days, 62 days, and 1 year after seedlings were transplanted. Results indicated that *C. geophilum* and *S. luteus* may negatively impact mycorrhizal colonisation and sporocarp formation in *L. japonica*. Sporocarp formation in *L. japonica* was positively correlated with conspecific mycorrhizal colonisation, but negatively correlated with the biomass of seedlings of *P. densiflora*. The co-occurring ECM fungi largely competed with *L. japonica*, resulting in various effects on mycorrhizal colonisation and sporocarp formation in *L. japonica*. A variety of mechanisms may be involved in the competitive interactions among the different ECM fungal species, including abilities to more rapidly colonise root tips, acquire soil nutrients, or produce antibiotics. These mechanisms need to be confirmed in further studies.

Keywords Sporocarps formation, Co-colonization, Ectomycorrhizal fungi, *Laccaria japonica*, *Pinus densiflora*

Introduction

Substantial research has been conducted on the evolutionary systematics, reproductive dynamics, ecological distribution, genetic diversity, genomes, and transcriptomes of ectomycorrhizal (ECM) fungi (Nara et al. 2003; Wang and Hall 2004; Lian et al. 2006; Fortin and Lamhamedi 2009; Xu et al. 2016; Parladé et al. 2017; Vaario et al. 2017). Nevertheless, environmental factors influencing the formation of ECM sporocarps are still poorly characterised. Such knowledge would be most useful for the cultivation of particular edible and commercially valuable ECM sporocarps, including both Ascomycetes (e.g. *Tuber* spp.) and Basidiomycetes such as *Amanita caesarea* (Scop.) Pers., *Boletus edulis* Bull., and *Tricholoma matsutake* (Ito & Imai) Singer (Wang and Hall 2004; Wang et al. 2012). Previous studies revealed that sporocarp formation in ECM fungi was affected by host genotype and soil properties (Last et al. 1984), and decreased with the reduction of carbohydrate supply to the roots of host plant (Högberg et al. 2001; Kuikka et al. 2003). Therefore, improving host growth may positively impact sporocarp formation in ECM fungi (Högberg et al. 2001; Nara et al. 2003). Soil nitrogen availability has been reported to significantly affect ECM sporocarp production, depending on the amount and duration of nitrogen additions (Velmala et al. 2014). Molecular studies have provided new insights into ECM sporocarp formation and development in *Tuber borchii* (Gabella et al. 2005), *Laccaria bicolor* (Martin et al. 2008), and *Tuber melanosporum* (Martin et al. 2010). Expression of the aquaporin genes JQ585592 and JQ585595 was highly upregulated during sporocarp development in *L. bicolor.* The aquaporin-mediated transmembrane transport of water and carbon dioxide played an important role in sporocarp development in *L. bicolor* as well (Xu et al. 2016). Despite these findings, research on sporocarp formation is still scarce because of the environmental factors involved in sporocarp formation, and the difficulty of producing sporocarps under controlled conditions (Smith and Read 2008; Wang et al. 2012; Sakamoto 2018).

In nature, forest trees are typically colonised by multiple species of ECM fungi (Tedersoo et al. 2007). ECM fungi are patchily distributed in the forest floor and influence one another's growth (Pickles et al. 2010). ECM fungi interact with a wide range of other soil fungi, including ECM fungi that co-occur on the same host plant (Villeneuve et al. 1991; Shaw et al. 1995; Velmala

et al. 2014). To date, a number of studies of ECM competition have been conducted. Hortal et al. (2008) found a negative correlation of the percentage of mycorrhizas between *Lactarius deliciosus* and *Rhizopogon roseolus* in pairwise interactions, which was also observed in interactions between species in the genus *Rhizopogon* (Kennedy et al. 2007, 2009, 2010). The environmental conditions seem to affect the competitive outcomes among ECM fungi. Erland and Finlay (1992) reported that the lower competitors of ECM fungi at low temperature became the equivalent or superior competitors at the high temperature due to the different colonising ability of ECM fungi to *Pinus sylvestris* roots at different temperatures. Moreover, in the interaction between *Piloderma croceum* and *Piloderma* sp. 1, *P. croceum* was dominant at low N concentrations, while *Piloderma* sp. 1 predominantly colonised the root tips of *Picea abies* at high N concentrations (Mahmood 2003; Kennedy 2010). However, whether sporocarp formation is influenced by co-colonising ECM fungal species is still poorly understood.

Laccaria japonica Popa & Nara is symbiotically associated with deciduous and coniferous trees in the Pinaceae, Fagaceae, and Salicaceae families in Japan (Vincenot et al. 2012, 2017). The sporocarps of *L. japonica* growing in nurseries, volcanic deserts, and forest edges usually appear from May to November in Japan (Vincenot et al. 2017). In our laboratory, sporocarps of *L. japonica* can be formed within 3 months under controlled conditions. Thus, *L. japonica* is an ideal experimental model for studying the mechanism of fruitbody formation. Additionally, the Japanese red pine *Pinus densiflora*, a common coniferous tree species in Japan, is widely cultivated for timber production and traditional gardening. Several hundred ECM fungal species have been reported from *P. densiflora* forests, such as the Ascomycetes *Cenococcum geophilum* Fr., *Helvella elastica*, and *H. macropus*, and the Basidiomycetes *Amanita* spp., *Boletus* spp., *Coltricia* spp., *Pisolithus* spp., *Laccaria* spp., *Lactarius* spp., *Russula* spp., and *Suillus* spp. (Yamada and Katsuya 2001; Satomura et al. 2003). Therefore, in this study, we used the symbiosis between *P. densiflora* and ECM fungi to investigate the effects of co-colonising ECM fungi on mycorrhizal colonisation and sporocarp formation in *L. japonica*.

Materials and Methods

ECM fungal cultures

Laccaria japonica (Lj) and three co-colonising ECM fungal species, *Cenococcum geophilum* (Cg), *Pisolithus* sp. (PS), and *Suillus luteus* (Sl), were used in this study. PS was previously known as *Pisolithus tinctorius* (Wu et al. 1999). The three co-colonising fungal species were chosen based on their abundance in *P. densiflora* forests and associations with a wide range of woody plants. More importantly, these three species can easily form ectomycorrhizae under laboratory conditions (Martin et al. 2002; Park et al. 2006; Douhan et al. 2007; Chen et al. 2015).

Samples of Lj, Cg, and PS were kindly provided by Dr. Kazuhide Nara (Graduate School of Frontier Sciences, University of Tokyo, Japan). Sl isolate was isolated from a sporocarp on Mount Fuji, Japan. The Lj host tree was *Salix reinii* and the host of the other three ECM fungal species was *P. densiflora*. All fungal cultures were maintained on modified Melin-Norkrans agar medium (Marx 1969).

Seedlings of *Pinus densiflora*

Seeds of *P. densiflora* were kindly provided by Dr. Maki Narimatsu (Iwate Prefectural Forestry Technology Center, Japan) and stored in a polyethylene bag in the dark at 4°C. Seeds were surface-sterilised by a 10-min submersion in 1% v/v sodium hypochlorite (NaClO), rinsed with deionised water, and germinated on autoclaved (180 min at 121°C) soil mixture in plastic pots (36 × 18 × 15 cm). The soil mixture was composed of a 2:1 v/v mixture of Shibanome soil, volcanic sand with pH 5.8–6.0 from Setogahara, Higashikurume, Japan, and forest soil from the University of Tokyo Tanashi Forest [see Chen et al. (2015) for details of the physicochemical properties of the soil mixture].

Preparation of seedlings colonised by ECM fungi

Non-mycorrhizal seedlings of *P. densiflora* were germinated approximately 2 to 3 months before the experiment started. These seedlings were then individually inoculated using fresh agar plugs (ca. 2 × 2 cm) containing fungal mycelia. Four types of ECM-colonised seedlings were prepared: Lj, Cg, PS, and Sl seedlings. Seedlings that were not inoculated (NM) served as the control. All seedlings were maintained in a temperature-controlled greenhouse at 20–25°C with a 16-h day and 8-h night light cycle.

Seedlings were randomly examined under a stereomicroscope to confirm mycorrhizal colonisation. Seedlings with mycorrhizal colonisation rates of more than 90% were chosen for

145 further study.

146 **Experimental set up**

147 Study details are summarised in Figure 1. Polypropylene pots (bottom diameter × upper
148 diameter × depth = 6 × 9 × 14 cm) were surface-sterilised with 70% v/v ethanol and filled with
149 510 g of autoclaved soil mixture. Four seedlings were planted in each pot according to the
150 following treatments: (1) 4 NM seedlings (control), (2) 1 Lj and 3 NM seedlings (Lj+NM), (3) 1
151 Lj, 2 NM, and 1 Cg seedling (Lj+Cg), (4) 1 Lj, 2 NM, and 1 PS seedling (Lj+PS), and (5) 1 Lj, 2
152 NM, and 1 Sl seedling (Lj+Sl). The Lj+Cg, Lj+PS, and Lj+Sl treatments represented the
153 co-colonising treatments. Co-colonising treatments had 15 replicates each and the control had nine
154 replicates. The soil in each pot was kept at approximately 80% soil moisture by adding tap water
155 at 2- to 3-day intervals during the experimental period.

156 ***In situ* seedling photosynthetic rates**

157 Seedling photosynthetic rates were measured using a portable LI-6400 photosynthetic
158 system (Li-COR, Lincoln, NE, USA) equipped with a conifer chamber (LI-6400-05, Li-COR). All
159 seedling needles were placed inside the conifer chamber under natural light with 400 ppm CO_2 at
160 25°C. Photosynthetic rates were first measured 30 days post-transplant, and then at approximately
161 30-day intervals until the end of the experiment. Three control pots and five pots for each
162 treatment were randomly chosen to measure photosynthetic rates at each time-point. One
163 randomly-selected formerly NM seedling from each pot, and all Lj, Cg, PS, and Sl seedlings in the
164 selected treatment pots were used for the measurements.

165 **Tracking the appearance of *L. japonica* primordia and sporocarps**

166 We defined the initial aggregate of mycelia that the distinct cap and stipe were not developed,
167 as primordia. The structure with distinct pileus and stipe was defined as sporocarp. Once Lj
168 primordia emerged at the soil surface, the numbers of primordia were recorded every 3–4 days
169 until the sporocarps appeared. Similarly, once the sporocarps appeared, sporocarp numbers in each
170 pot were recorded, and sporocarps (length: 5–10 mm) were collected every 3–4 days until the end
171 of the experiment. The collected sporocarps were dried in an oven at 60°C for 48 h and weighed.

172 **Quantifying ECM colonisation rates and determining seedling biomass**

173 Samples were harvested at three time-points for examination: (1) 45 days after seedlings

were transplanted (before Lj primordia appeared), (2) 62 days post-transplant (when Lj sporocarps first appeared), and (3) 1 year post-transplant. Seedlings in three control pots and five pots for each treatment were harvested at each sampling period. All seedlings were used to determine mycorrhizal colonisation rates and biomass. The roots of each seedling were separated from the shoot and washed gently in tap water. All root tips were then examined under a stereomicroscope to determine the mycorrhizal colonisation rate.

Colonisation rate (%) = number of mycorrhizal root tips / total number of root tips of whole seedling × 100.

After examining the roots, the roots and shoots of all seedlings were dried at 60°C for 48 h to determine biomass.

Quantifying *L. japonica* mycelia in soil and mycorrhizal root tips using real-time PCR

Sampling: To examine the development of Lj mycelia, we sampled the soil and Lj-mycorrhizal root tips of formerly NM seedlings from each pot at 45 days, 62 days, and 1 year after seedlings were transplanted. The soil in each pot was mixed well after seedlings were removed, and approximately 5 g of soil was collected from each pot. Samples were immediately processed in an FDS-1000 vacuum freeze dryer (Tokyo Rikakikai Co. Ltd, Japan) and stored at -30°C. In addition, 10 mycorrhizal root tips with Lj were sampled from each formerly NM seedling, dried using silica-gel desiccant at 4°C, and stored at -30°C.

DNA extraction: DNA from 0.1 g of dry soil per sample was extracted using the ISOIL for Beads Beating kit (Nippon Gene Co. Ltd., Fukuyama, Japan) according to the manufacturer's instructions. Ten dried mycorrhizal root tips per sample were used for DNA extraction, which was carried out using a modified cetyl-trimethylammonium bromide (CTAB) method (Lian et al. 2003).

Designing species-specific Lj primers: The species-specific primers La03f (ATGAGCCTGATGTGGCTGT) and La03r (TGGCAATGAATGGAAAGC) in the rDNA internal transcribed spacer (ITS1) region were designed using Primer Express 5.0 software (Applied Biosystems, Foster City, CA, USA). To increase primer specificity to Lj, we changed several nucleotides in each primer sequence. The size of PCR-amplified products was 147 bp. We confirmed that this primer pair was specific to Lj, as they did not amplify Cg, PS, and Sl DNA, or

DNA extracted from control soil samples (Supplementary Fig. S1).

Real-time PCR (RT-PCR): We prepared a standard curve for Lj abundance to quantify Lj mycelia in the soil. First, 0.02 g of freeze-dried mycelia was collected from a 1-month-old Lj colony growing on cellophane. The mycelia were thoroughly mixed with 0.98 g of freeze-dried soil using a mixer-mill machine (Retsch GmbH & Co. KG, Tokyo, Japan). DNA was extracted from the mixture as previously described for other soil samples. We performed a 10-fold serial dilution of the extracted sample from 10^0 to 10^{-5}, and quantified DNA at each dilution step using RT-PCR. A standard curve was also prepared for Lj abundance in mycorrhizal root tips. DNA from 11.40 mg of freeze-dried mycelia of the 1-month-old Lj colony was extracted using the modified CTAB method and diluted from 10^0 to 10^{-5} for quantification by RT-PCR. Relative biomass of Lj mycelia in the soil and mycorrhizal root tips was estimated by interpolating the Cq value of each sample in the standard curves (Parladé et al. 2007; De la Varga et al. 2012). For RT-PCR, DNA samples were amplified in a Thermal Cycler Dice® TP800 RT-PCR system (Takara Co. Ltd., Tokyo, Japan) using the THUNDERBIRD® SYBR® qPCR Mix (Toyobo Co. Ltd., Osaka, Japan). Each RT-PCR reaction sample (20 µl) contained 7.4 µl ddH_2O, 0.3 µl (20 µM) forward (La03f) and reverse (La03r) primers, 10 µl THUNDERBIRD® SYBR® qPCR Mix, and 2 µl DNA solution. PCR conditions were: 95°C for 1 min, 40 cycles of 95°C for 15 s and 60°C for 30 s, and a final dissociation period of 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. Data were processed with the Thermal Cycler Dice® RT-PCR Software Version 4.0. Results were converted to $mg \cdot g^{-1}$ of soil or root tip dry weight.

Statistical analysis

Results are presented as mean values ± standard deviation and mean ± 95% confidence interval in the tables and figures, respectively. All datasets were tested for data normality and homogeneity of variance.

Non-parametric pairwise comparisons among the different mycorrhizal treatments of the numbers of primordia and sporocarps, sporocarp dry weight, seedling biomass and photosynthetic rates, total number of Lj-colonised root tips, and biomass of Lj mycelia in mycorrhizal root tips were performed using the Wilcoxon rank sum test ($n = 5$, $\alpha = 0.05$). *P*-values were corrected using the Benjamini–Hochberg method. The Wilcoxon rank sum test was also used to compare, within

the same mycorrhizal treatment, the biomass of Lj mycelia in mycorrhizal root tips collected 45 and 62 days after seedlings were transplanted. Similarly, the mycorrhizal colonisation rates of the four ECM fungal species were compared at 45 days, 62 days and 1 year post-transplant within the same mycorrhizal treatments (n = 5).

Seedling biomass and photosynthetic rates of formerly NM seedlings were compared between each mycorrhizal treatment and the control using the non-parametric Steel test (R-Package 'nparcomp') (n = 3–5).

The association between total number of Lj-colonised root tips and numbers of Lj primordia and sporocarps, and the association between biological parameters of seedlings of *P. densiflora* and numbers of Lj primordia and sporocarps, were tested using non-parametric Spearman's rank correlation. All statistical analyses were performed with R 3.5.1 software (R Core Team 2018).

Results

L. japonica primordium and sporocarp formation

The Lj primordia were firstly observed in the Lj+NM treatment 50 days after seedlings were transplanted. Thereafter, Lj primordia were observed in the Lj+PS and Lj+Cg treatments at 51 days, and in the Lj+Sl treatment at 63 days. Lj sporocarps initially appeared in the Lj+PS, Lj+NM, Lj+Cg, and Lj+Sl treatments at 62, 63, 65, and 72 days post-transplant, respectively (Table 1). PS and Sl primordia and sporocarps were not observed during the study. Primordia and sporocarps were also not observed in control NM pots.

Numbers of Lj primordia were recorded until the first sporocarp appeared in the Lj+PS treatment at 62 days post-transplant. Primordia were not observed in the Lj+Sl treatments. A low number of primordia was observed in the Lj+Cg treatment, although the difference with the Lj+NM and Lj+Cg treatments was not statistically significant (Fig. 2a). Sporocarp abundance was recorded during the 1-year study period. Sporocarp number and biomass was lowest in the Lj+Sl treatment, and significantly lower than sporocarp abundance in the Lj+PS treatment (n = 5, $P < 0.05$, pairwise Wilcoxon rank sum test; Fig. 2b, 2c).

Growth and photosynthetic rates of seedlings of *P. densiflora*

No significant differences in seedling root or shoot growth were observed among the Lj+NM, Lj+Cg, Lj+PS, and Lj+Sl treatments at 45 and 62 days after seedlings were transplanted (Fig. 3a, b). One year post-transplant, shoot growth in the Lj+Sl treatment was significantly greater than in the other treatments (n = 5, $P < 0.05$, pairwise Wilcoxon rank sum test; Fig. 3a), but no significant differences in root growth were found (Fig. 3b). Shoot and root growth in the control was significantly lower than in the Lj+NM, Lj+Cg, Lj+PS, and Lj+Sl treatments 1 year post-transplant (n = 3–5, $P < 0.05$, Steel test; Supplementary Table S1).

Significantly lower photosynthetic rates were observed for NM seedlings in control pots compared to formerly NM seedlings in the mycorrhizal treatment pots at 62 days and 1 year post-transplant (n = 3–5, $P < 0.05$, Steel test; Supplementary Fig. S2a). Photosynthetic rates of the formerly NM seedlings were not significantly different among the mycorrhizal treatments. No significant differences in the photosynthetic rates of the Lj, Cg, PS, and Sl seedlings were found among the mycorrhizal treatments at all sampling times (Supplementary Fig. S2b, c).

Mycorrhizal colonisation of seedlings

During the study period, mycorrhizal roots were not observed in control seedlings. In all co-colonising treatments, the roots of all NM, Lj, Cg, PS, and Sl seedlings were colonised by both Lj and the corresponding ECM fungal species at 45 days after seedlings were transplanted (Fig. 4).

For the Lj+NM treatment, the roots of the formerly NM seedlings and Lj inoculated seedlings were almost completely colonised by Lj, with colonisation rates of more than 88% 45 days post-transplant. But the colonisation rate decreased 1 year post-transplant (Fig. 4a, b). For the Lj+Cg treatment, Lj and Cg colonisation rates in the roots of the formerly NM and Lj seedlings did not differ markedly at 45 and 62 days post-transplant. One year post-transplant, the roots of seedlings were dominantly colonised by Cg (Fig. 4c, d). For the Cg seedlings, the roots were dominantly colonised over the course of the experiment (colonisation rate > 77%) (Fig. 4e). For the Lj+PS treatment, Lj colonisation rates (> 67%) in the roots of the formerly NM and Lj seedlings were significantly higher than those of PS (< 28%) (n = 5, $P < 0.05$, Wilcoxon rank sum test; Fig. 4f, g). In the roots of the PS seedlings, Lj colonisation rate was significantly higher than that of PS at 45 post-transplant but significantly lower 1 year post-transplant (n = 5, $P < 0.05$,

Wilcoxon rank sum test; Fig. 4h). For the Lj+Sl treatment, in the formerly NM and Sl seedlings, the roots were dominantly colonised by Sl (rate > 72%) at 45 days and 62 days post-transplant but the colonisation rates of Lj and Sl were not significantly different at 1 year post-transplant (n = 5, $P > 0.05$, Wilcoxon rank sum test; Fig. 4i, k). In the roots of Lj seedlings, Lj and Sl colonization rates were not significant though the Sl rate was a little higher than Lj at 62 days post-transplant (Fig. 4j).

The total numbers of Lj root tips in each pot in the different mycorrhizal treatments are shown in Fig. 5. The number of Lj root tips in the Lj+Cg and Lj+Sl treatments was significantly lower than in the Lj+NM and Lj+PS treatments at 45 and 62 days post-transplant (n = 5, $P < 0.05$, pairwise Wilcoxon rank sum test). One year post-transplant, the number of Lj root tips in the Lj+Sl treatment was significantly lower than in the Lj+PS treatment (n = 5, $P < 0.05$, pairwise Wilcoxon rank sum test).

L. japonica mycelial biomass in soil and mycorrhizal roots

Lj mycelium was not detected in most soil samples at 45 and 62 days after seedlings were transplanted. By contrast, Lj mycelium was detected in the mycorrhizal root tips of formerly NM seedlings. No significant differences in Lj mycelial biomass in the mycorrhizal root tips of formerly NM seedlings were observed among the different treatments at 45 and 62 days post-transplant (Supplementary Table S2). The Lj mycelial biomass in mycorrhizal root tips at 62 days post-transplant was higher than mycelial biomass at 45 days post-transplant (Supplementary Table S2).

Associations between primordium and sporocarp abundance and the total numbers of *L. japonica* root tips, and growth of *P. densiflora* seedlings

Primordium abundance was positively and significantly correlated with the total numbers of Lj root tips at 45 days (r = 0.77, $P < 0.01$) and 62 days (r = 0.83, $P < 0.01$) post-transplant (Table 2). Primordium abundance was also negatively and significantly correlated with total net increase in root dry weight (45 days post-transplant, r = -0.48, $P < 0.05$), and with the photosynthetic rate of NM seedlings (62 days post-transplant, r = -0.56, $P < 0.05$; Table 2).

Similarly, sporocarp abundance was positively and significantly correlated with the total numbers of Lj root tips at 45 days (r = 0.56, $P < 0.05$) and 62 days (r = 0.59, $P < 0.01$)

post-transplant (Table 2). In addition, sporocarp abundance was negatively and significantly correlated with the total net increase in both shoot ($r = -0.60$, $P < 0.01$) and root ($r = -0.80$, $P < 0.01$) dry weight 1 year post-transplant (Table 2).

Discussion

Effects of interspecific competition on ectomycorrhizae formation

In this study, we investigated the effects of co-colonisation between *L. japonica* and other ECM fungal species in the roots of *P. densiflora* on host plant growth and sporocarp formation. The presence of other co-colonising species affected primordium and sporocarp formation by *L. japonica*, likely as the result of competition among the different ECM fungal species. The number of primordia and sporocarps produced depended on the mycorrhizal colonisation rate of *L. japonica*. Of the three ECM fungal species used to co-colonise *P. densiflora* in this study, *S. luteus* negatively affected sporocarp formation by *L. japonica*, *C. geophilum* showed a similar trend and *Pisolithus* sp. had no effect.

Cenococcum geophilum has a global distribution range and is the most abundant fungal species in natural communities of ectomycorrhizae (Koide et al. 2005; Pickles et al. 2012). Our results revealed that in the treatment with both *L. japonica* and *C. geophilum*, the roots of *P. densiflora* were predominantly colonised by *C. geophilum* 1 year after seedlings were transplanted (Fig. 4c–e), suggesting that *C. geophilum* was more successful than *L. japonica* in colonising the roots of *P. densiflora*, which decreased sporocarp formation in *L. japonica*. Our results confirmed previous findings that *C. geophilum* is often the dominant ECM fungal species in pairwise interactions with other fungal species (Shaw et al. 1995; Dalong et al. 2011).

Pisolithus tinctorius forms extensive rhizomorphs that promote nutrient and water transport to the roots of the host plant (Cairney and Smith 1992; Cairney and Chambers 1997). However, mycorrhizal colonisation appears to be impeded by rhizomorphs (Wu et al. 1999). In general, *P. tinctorius* are regarded as poor competitors of other ECM fungi as they are unable to sustain the enhancement of growth in host seedlings under cool conditions (McAfee and Fortin 1986; Cairney and Chambers 1997; Garcia-Barreda et al. 2015). Wu et al. (1999) examined interactions among three ECM fungal species, *P. tinctorius*, *S. luteus*, and an unidentified species termed “Tanashi 01”.

In the experiments with both *S. luteus* and *P. tinctorius*, the presence of the other species did not affect the colonisation rate of each species but inhibited mycelial development. By contrast, mycorrhizal colonisation and growth in *P. tinctorius* were inhibited by Tanashi 01. In our study, *L. japonica* dominated the mycorrhizae in the roots of non-inoculated seedlings in the presence of *Pisolithus* sp. (formerly known as *P. tinctorius*) (Lj+PS treatment) throughout the experimental period (Fig. 4f), which indicated that *Pisolithus* sp. was out-competed by *L. japonica*. Additionally, other species of *Pisolithus* such as *P. arhizus* have been shown to be less competitive in interspecific interactions (Parladé and Alvarez 1993).

Suillus luteus has a higher capacity for decomposing organic substrates than other mycorrhizal fungal species (Dighton et al. 1987) and has been shown to significantly improve the growth of its host seedlings (Lu et al. 2016). Accordingly, we recorded significantly higher aboveground seedling biomass in the presence of *S. luteus* (Lj+Sl treatment) 1 year after seedlings were transplanted (Fig. 3a). This may be why *S. luteus* is able to colonise seedling roots at a higher rate than *L. japonica*. The congeneric *S. granulatus* also seems to more efficiently access limiting resources for host plant growth, and to transfer these resources to its host, thus, increasing host plant biomass (Kipfer et al. 2012).

To date, little is known about colonisation competition among different ECM fungal species. ECM fungal species have different capacities for nutrient uptake and transfer, and also differ in their ability to promote host growth (Burgess et al. 1993, 1994; Agerer 2001). Differences in the ability to rapidly colonise root tips or acquire soil nutrients may be attributed to nutrient competition between paired ECM fungal species (Kennedy 2010). Moreover, the ways that extraradical hyphae emanate and explore their surroundings are different among ECM fungal species (Read 1992; Colpaert et al. 1992; Thomson et al. 1994; Agerer 2001). These interspecific differences may affect competition in root colonisation among ECM fungal species. In our study, the four species of ECM fungi had hyphae of different exploration types, ranging from short- to long-distance (Agerer 2001), representing differences in foraging strategies. In preliminary experiments, mycelial growth rates in *Pisolithus* sp. and *S. luteus* were significantly higher than in *L. japonica* and *C. geophilum* in mycelial culture. In the first month, growth rates of *L. japonica* and *C. geophilum* were similar, but *C. geophilum* grew faster thereafter. While preparing the

mycorrhizal seedlings of *P. densiflora*, we found that ectomycorrhizae formation in *L. japonica* and *S. luteus* was significantly faster than in plants inoculated by *C. geophilum* and *Pisolithus* sp (unpublished data). However, as co-colonisers, *C. geophilum* and *S. luteus* significantly inhibited mycorrhizal colonisation by *L. japonica*. In turn, *L. japonica* significantly inhibited ectomycorrhizae formation in *Pisolithus* sp. (Fig. 4). These results suggest that the intrinsic mycelial growth rate and ectomycorrhizae formation capability of each ECM fungal species may not have a strong influence on competitive outcomes among co-colonising species.

Mycorrhizal fungi can produce antimicrobial substances that defend against rhizosphere microorganisms (Zhang et al. 2011; Mohan et al. 2015; Mateos et al. 2017). Therefore, the inhibition of ectomycorrhizae formation by co-existing ECM fungal species might be due in part to the production of antimicrobial substances.

Finally, it is possible that the soil mineral and nutrient content was unfavourable for mycorrhizae formation and seedling growth over the 1-year study period. The number of mycorrhizal root tips containing *L. japonica* and *S. luteus* decreased 1 year after seedlings were transplanted. By contrast, the number of mycorrhizal root tips containing *C. geophilum* increased after 1 year. It has been previously reported that *C. geophilum* develops well in soils with poor nutrient conditions (Frankland and Harrison 1985; McAfee and Soil 1989; Villeneuve et al. 1991). Therefore, the soil nutrient content may have affected interactions among the ECM fungal species.

Relationship between primordium and sporocarp formation in *L. japonica* and host growth

Our results revealed that colonisation by ECM fungi significantly increased host seedling biomass, consistent with findings from previous studies (Smith and Read 2008). Further, the rate of sporocarp formation may influence host plant biomass. The biomass of seedlings of *P. densiflora* decreased with increases in sporocarp number (Figs. 2 and 3, Table 2). Previous studies have shown that sporocarp abundance decreased significantly when hosts were artificially defoliated or when host trees were girdled, again supporting our findings (Högberg et al. 2001; Kuikka et al. 2003). A study using ^{14}C-labelled photosynthates revealed that recently-produced photosynthates were mainly transferred to the sporocarps of *L. japonica* (reported in this study as *Laccaria amethystina*; Teramoto et al. 2012).

Factors influencing primordium and sporocarp formation in *L. japonica*

Primordium abundance was significantly and positively correlated with the total numbers of *L. japonica* root tips 45 and 62 days before sporocarp formation (Table 2), whereas sporocarp abundance had a weak positive correlation with the total numbers of *L. japonica* root tips 1 year post-transplant (Table 2). During the experiment, we observed the formation of numerous primordia, but most of them did not grow further and only few developed into sporocarps. This phenomenon was also reported by Teramoto et al. (2012). Mycorrhizal colonisation rates appeared to directly influence primordium formation, but the development of primoridia to sporocarps may be dependent on the nutrients available and the presence of competitors. Biotic factors such as interspecific competition and abiotic factors such as moisture levels, substrate pH, temperature, and nutrients have been shown to influence sporocarp development (Godbout and Fortin 1990; Kües and Liu 2000; Kennedy 2010).

Effects of interspecific competition on the mycelial biomass of *L. japonica* in soil and mycorrhizae

Our results indicate that the mycelial biomass of *L. japonica* in soil and mycorrhizal root samples was not influenced by co-colonising ECM fungal species, which are consistent with previous reports (Hortal et al. 2008; De la Varga et al. 2012). The DNA of *L. japonica* was not detected in most of the samples at 45 and 62 days after seedlings were transplanted (Supplementary Table S2), indicating that there was little extraradical mycelium in the soil at those sampling time points. It has been reported that *Laccaria* spp. and some *Lactarius* spp. have hyphae classified as medium- or short-distance exploration types (Agerer 2001). The extraradical soil mycelia of *Lactarius deliciosus* were shown to be significantly affected by sampling time (Parladé et al. 2007). Sampling time may be an important consideration for measuring the mycelial biomass of *L. japonica* in soil or mycorrhizal root tips. The mycorrhizal colonisation rate of *L. japonica* in non-inoculated seedlings ranged from 23% to 88% in this study. In other words, most of the seedlings hosted mycorrhizae with *L. japonica*. This may be why we did not find differences in the mycelial biomass of *L. japonica* in mycorrhizal root tips among seedlings in the different treatments.

Conclusions

The results of this study showed that the ECM fungal species co-colonising the roots of seedlings of *P. densiflora* had different effects on sporocarp formation in *L. japonica*. *Cenococcum geophilum* and *S. luteus* appeared to have a negative impact on the mycorrhizal colonisation rate and sporocarp formation in *L. japonica*, but *Pisolithus* sp. had no effect. Sporocarp formation in *L. japonica* was positively correlated with its own mycorrhizal colonisation rate, but negatively correlated with seedling biomass. The coexisting ECM fungus might have more influence on mycorrhizal colonisation and sporocarp formation rather than extraradical mycelia of *L. japonica* in this study.

In our study, investigations were carried out in controlled greenhouse systems. We recommend further investigations in the field environment to fully understand the competitive mechanisms of ECM fungal species.

Competing interests

The authors declare no conflict of interests.

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640

Figure Legends

Figure 1. Schematic diagram of the experimental setup. Seedlings of *Pinus densiflora* were used in all treatments. Each pot (circle) contained four seedlings. NM: non-mycorrhizal seedling, Lj: seedling colonised by *Laccaria japonica*, Cg: seedling colonised by *Cenococcum geophilum*, PS: seedling colonised by *Pisolithus* sp., Sl: seedling colonised by *Suillus luteus*, Con: control.

Figure 2. (a) Total number of primordia of *Laccaria japonica* observed in each treatment at 62 days after seedlings were transplanted. *L. japonica* sporocarps were first observed at 62 days post-transplant. (b) Total number and (c) total dry weight of sporocarps for each treatment 1 year after seedlings were transplanted. Lj+NM: treatment with non-mycorrhizal seedlings and seedlings colonised by *L. japonica*; Lj+Cg: treatment with non-mycorrhizal seedlings and seedlings colonised by *L. japonica* and *Cenococcum geophilum*; Lj+PS: treatment with non-mycorrhizal seedlings and seedlings colonised by *L. japonica* and *Pisolithus* sp.; Lj+Sl: treatment with non-mycorrhizal seedlings and seedlings colonised by *L. japonica* and *Suillus luteus.* Different letters indicate significant differences among treatments (n = 5, $P < 0.05$, Wilcoxon rank sum test, *P*-value adjusted using the Benjamini–Hochberg [BH] method).

Figure 3. Total net increase in (a) shoot and (b) root biomass of all seedlings of *Pinus densiflora* in each pot at 45 days, 62 days, and 1 year after seedlings were transplanted. Each pot contained four seedlings. NM: non-mycorrhizal seedling, Lj: seedling colonised by *Laccaria japonica*, Cg: seedling colonised by *Cenococcum geophilum*, PS: seedling colonised by *Pisolithus* sp., Sl: seedling colonised by *Suillus luteus.* Values are reported as means ± standard deviation (SD; n = 5). Different letters indicate significant differences among treatments (n = 5, $P < 0.05$, Wilcoxon rank sum test, *P*-value adjusted using the BH method).

Figure 4. Changes of mycorrhizal colonization rate of different ECM fungi in formerly NM seedlings and in seedlings colonised by *L. japonica* (Lj), *C. geophilum* (Cg), *Pisolithus* sp. (PS), *S. luteus* (Sl). For treatments : see Fig. 1. Colonisation rates were measured at 45 days, 62 days, and

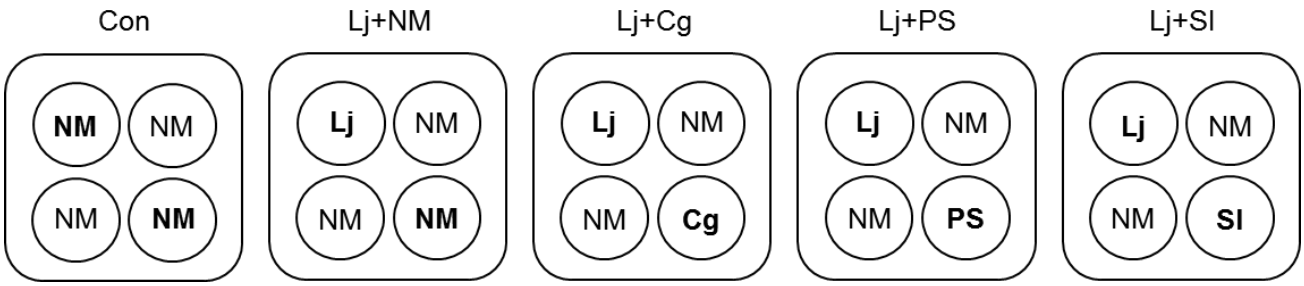
1 year after seedlings were transplanted. Values are represented as mean ± SD (n = 5). The Wilcoxon rank sum test was used to test for differences in colonisation rate between Lj and the other fungal species, *$P < 0.05$.

Figure 5. Number of root tips of *L. japonica* on *P. densiflora* roots at 45 days, 62 days, and 1 year after seedlings were transplanted. For treatments: see Fig. 1. Values are represented as mean ± SD (n = 5). Pairwise comparisons using the Wilcoxon rank sum test were conducted to test for differences among treatments. Letters indicate significant differences ($P < 0.05$, *P*-value adjusted using the BH method).

Supplementary Figure S1. Agarose gel electrophoresis of PCR products from amplification using the primer La03f/03r. Lane M: 100 bp ladder marker. Lane 1: DNA extracted from mycelia of *Laccaria japonica*. Lane 2: negative control. Lane 3: DNA extracted from mycelia of *Cenococcum geophilum*. Lane 4: DNA extracted from mycelia of *Pisolithus* sp. Lane 5: DNA extracted from mycelia of *Suillus luteus*. Lane 6: DNA extracted from soil control. Lane 7: DNA extracted from non-mycorrhizal root tips.

Supplementary Figure S2. The net photosynthetic rates of (a) non-mycorrhizal seedlings (NM), (b) seedlings colonised by *Laccaria japonica* (Lj), and (c) seedlings colonised by *Cenococcum geophilum* (Cg), *Pisolithus* sp. (PS), and *Suillus luteus* (Sl) at 45 days, 62 days, and 1 year after seedlings were transplanted into pots. Each pot contained four seedlings. Con: control with only NM seedlings; Lj+NM: treatment with NM seedlings and Lj-colonised seedlings; Lj+Cg: treatment with NM seedlings and Lj- and Cg-colonised seedlings; Lj+PS: treatment with NM seedlings and Lj- and PS-colonised seedlings; Lj+Sl: treatment with NM seedlings and Lj- and Sl-colonised seedlings. Values are represented as mean ± SD (n = 3–5). Pairwise comparisons using the Wilcoxon rank sum test were conducted to test for differences among treatments. Letters indicate significant differences ($P < 0.05$, *P*-value adjusted using the BH method). The Steel test was also used to test for differences between the control and each treatment, *$P < 0.05$.

Fig 1


Con
NM
NM
NM
NM
Lj+NM
Lj
NM
NM
NM
Lj+Cg
Lj
NM
NM
Cg
Lj+PS
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NM
NM
PS
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Lj
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NM
Sl

Fig 2

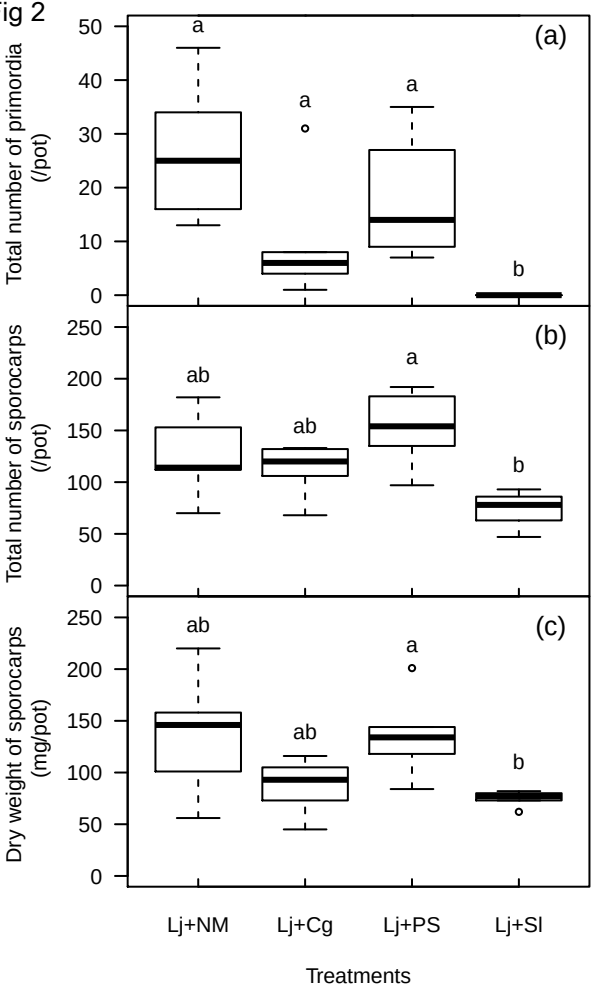
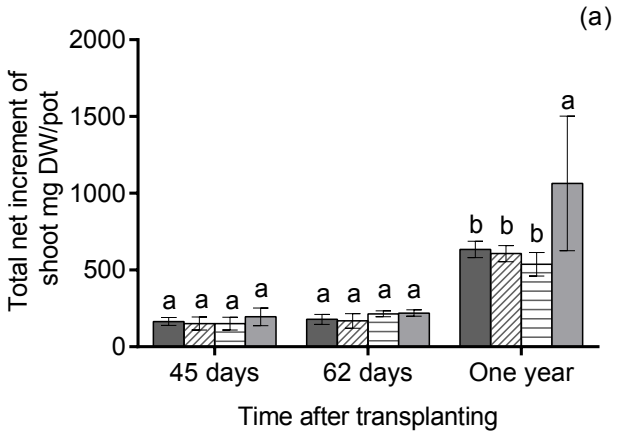
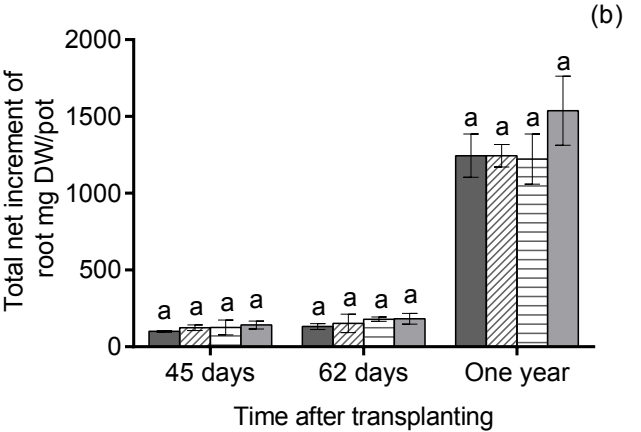

(a)
Total number of primordia (/pot)
50
40
30
20
10
0
a
a
a
b
(b)
Total number of sporocarps (/pot)
250
200
150
100
50
0
ab
ab
a
b
(c)
Dry weight of sporocarps (mg/pot)
250
200
150
100
50
0
ab
ab
a
b
Lj+NM
Lj+Cg
Lj+PS
Lj+Sl
Treatments

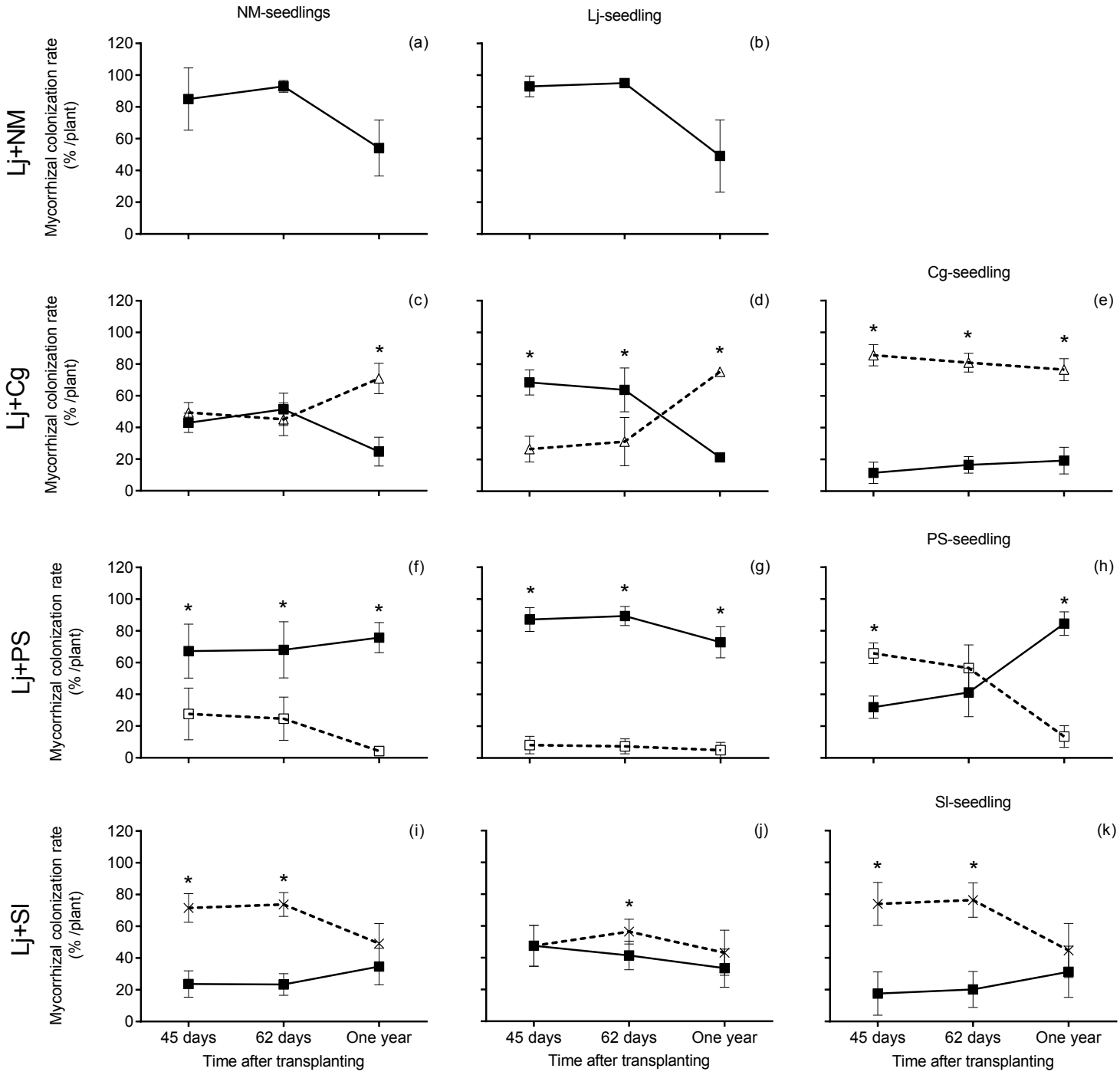
Fig 3





Lj+NM Lj+Cg Lj+PS Lj+Sl

Fig 4


NM-seedlings
Lj-seedling
Cg-seedling
PS-seedling
Sl-seedling
Lj+NM
Lj+Cg
Lj+PS
Lj+Sl
Mycorrhizal colonization rate (% /plant)
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
45 days
62 days
One year
Time after transplanting
Lj
Cg
PS
Sl

Revised Fig 5

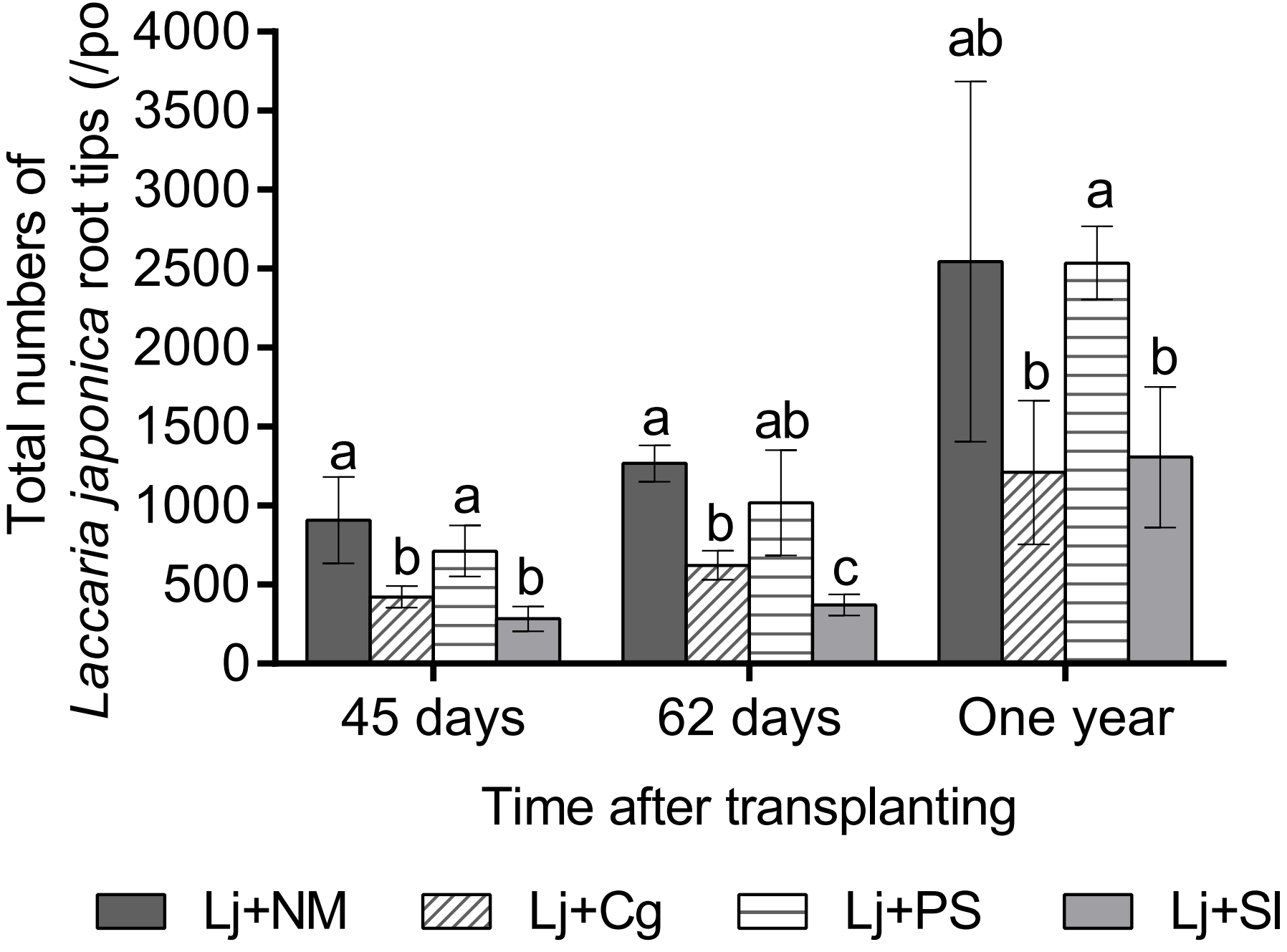


Table 1

Table 1. The time of first appearance of primordia and sporocarps of *Laccaria japonica* in the different treatments

Treatments	First appearance (days)/after transplanting	
	Primordia	Sporocarps
Con	None	None
Lj+NM	50	63
Lj+Cg	51	65
Lj+PS	51	62
Lj+Sl	63	72

NM: non-mycorrhizal seedling, Lj: seedling colonised by *Laccaria japonica*, Cg: seedling colonised by *Cenococcum geophilum*, PS: seedling colonised by *Pisolithus* sp., Sl: seedling colonised by *Suillus luteus*, Con: control.

Revised Table 2

Table 2. Correlations between the number of primordia and sporocarps of *Laccaria japonica* and the biological parameters of seedlings of *Pinus densiflora* at 45 days, 62 days, and 1 year after seedlings were transplanted

		Biological parameter	45 days		62 days		One year	
			Spearman's rho coefficient	*P* value	Spearman's rho coefficient	*P* value	Spearman's rho coefficient	*P* value
The total numbers of primordia	n=20	Total numbers of Lj root tips /pot	**0.77**	**0.00**	**0.83**	**0.00**	-	-
		Total net increment dry weight of shoot/pot	-0.08	0.73	-0.29	0.22	-	-
		Total net increment dry weight of root/pot	**-0.48**	**0.03**	-0.28	0.23	-	-
		Net photosynthetic rate of formerly NM-seedlings	-0.16	0.51	**-0.56**	**0.01**	-	-
		Net photosynthetic rate of Lj-seedling	0.25	0.30	-0.33	0.15	-	-
	n=5	Net photosynthetic rate of Cg-seedling	0.20	0.78	-0.20	0.78	-	-
		Net photosynthetic rate of PS-seedling	-0.10	0.95	0.00	1.00	-	-
		Net photosynthetic rate of Sl-seedling	-	-	-	-	-	-
The total numbers of sporocarps	n=20	Total numbers of Lj root tips /pot	**0.56**	**0.01**	**0.59**	**0.00**	0.25	0.29
		Total net increment dry weight of shoot/pot	-0.22	0.36	-0.11	0.64	**-0.60**	**0.00**

Table 2. Continued

		Biological parameter	45 days		62 days		One year	
			Spearman's rho coefficient	*P* value	Spearman's rho coefficient	*P* value	Spearman's rho coefficient	*P* value
The total numbers of sporocarps	n=20	Total net increment dry weight of root/pot	-0.11	0.63	0.05	0.85	**-0.80**	**0.00**
		Net photosynthetic rate of formerly NM-seedlings	0.02	0.94	-0.35	0.13	-0.16	0.50
		Net photosynthetic rate of Lj-seedling	-0.01	0.97	-0.24	0.32	-0.25	0.29
	n=5	Net photosynthetic rate of Cg-seedling	-0.40	0.52	-0.50	0.45	0.10	0.95
		Net photosynthetic rate of PS-seedling	-0.40	0.52	0.70	0.23	-0.10	0.95
		Net photosynthetic rate of Sl-seedling	0.70	0.23	-0.70	0.23	0.30	0.68

NM: non-mycorrhizal seedling, Lj: seedling colonised by *Laccaria japonica*, Cg: seedling colonised by *Cenococcum geophilum*, PS: seedling colonised by *Pisolithus* sp., Sl: seedling colonised by *Suillus luteus*, Con: control.

Supplementary Fig S1

Supplementary Fig S2

Supplementary Table S1